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Nucleoli in a pronuclei-stage mouse embryo are represented by major satellite DNA of interconnecting chromosomes

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Objective: To investigate the arrangement of chromosomes within pronuclei-stage mouse zygotes.

Design: In vitro study.

Setting: Academic medical center.

Patient(s): None.

Intervention(s): None.

Main Outcome Measure(s): Location of major α-satellite DNA, centromeres, and telomeres, and relative location of chromosomes.

Result(s): Chromosomes appeared to be oriented inward by centromeres and to be interconnected by major α-satellite DNA, which appeared to be the sole DNA component of the nucleoli. This chromosomal arrangement persisted throughout interphase. Chromosomal painting failed to identify chromosomal ordering within pronuclei.

Conclusion(s): Pronuclear nucleoli are represented by α-satellite sequences of interconnecting chromosomes that hold all chromosomes together during interphase. Chromosomes within the pronucleus are randomly positioned relative to each other. (Fertil Steril® 2000;73:366–71. ©2000 by American Society for Reproductive Medicine.)

Key Words: Zygote, nucleoli, α-satellite, centromeres, telomeres

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Evidence is accumulating that the interphase nucleus is highly structured (1, 2), and it also is believed that at least one of the levels of nuclear organization is represented by nonrandom positioning of chromosomes relative to each other (3–6). However, because of the difficulties involved with the simultaneous resolution of several chromosomes during interphase, data collected so far have failed to provide a convincing argument to support or refute nonrandom chromosomal location. At the same time, data on nonrandom chromosomal connection and orientation throughout the cell cycle are more compelling. Most of these data are based on the position and orientation of centromeres relative to the nucleolus, and to each other, during interphase (7, 8).

Moreover, it recently was demonstrated that in humans, during prometaphase, the centromeric ends of the chromosomes are juxtaposed to each other and homologous chromosomes are located on the opposite sides of the rosette in an ordered, antiparallel fashion (9). In addition, in humans, during the pronuclei stage, it is not uncommon to see the alignment of nucleoli between pronuclei at syngamy indicating some sort of even more complex chromosomal order. Remarkably, it was demonstrated recently that this alignment may serve as a prognostic criterion for embryo quality (10). In the present study, we determined the details of chromosomal arrangement in mouse embryos; this identifies chromosomal behavior in a zygote and also may explain the nucleoli alignment observed in human zygotes.

MATERIALS AND METHODS

Animals

Four or five 6- to 10-week-old female mice (C57B6 × DBA/2)F1 were used in each of a total of 25 experiments. Each particular experiment was replicated at least three times. Superovulation was induced by the injection of 10 IU of pregnant mare serum gonadotropin followed by 10 IU of hCG 48 hours later. For fertilization, the female mice were caged with male mice, and those with a vaginal plug 12 hours later were killed using CO_2 inhalation. After this, zygotes were recovered from the oviduct and cultured further in plain M16 media in an atmosphere of 5% CO_2 in air. In each experiment, 100–150 zygotes were used.

Approximately 27 hours after the administration of hCG, zygotes were transferred into M16 media containing 10 μg/mL of okadaic acid (OA). They were cultured further in an atmosphere of 5% CO_2 for approximately 50 minutes, and those that contained pronuclei visible under the dissecting microscope were subjected to fixation.

After short hypotonic treatment with 0.9% potassium chloride, zygotes were transferred into a mixture of acetic acid and methanol (1:3) and subsequently dropped onto glass slides and allowed to air-dry. Some slides were stained with 7% Giemsa, whereas others were processed further for chromosomal painting or fluorescence in situ hybridization (FISH) with telomeric or centromeric probes. Some zygotes were fixed in 2% formaldehyde and processed further for fluorescent microscopy.

Spectral Karyotyping

Spectral karyotyping in the mouse is a genome-wide cytogenetic screening method that uses 20 uniquely labeled chromosome-specific FISH probes, epifluorescence microscopy, digital imaging, and Fourier spectroscopy in a protocol that has been described in detail elsewhere (11, 12). In brief, the metaphase cells were pretreated by equilibration in 2× SSC, digested with ribonuclease A (0.1 mg/mL) and pepsin (10 μg/mg), fixed in 1% formaldehyde, denatured in 70% formamide/2× SSC (2 minutes at 85°C), dehydrated in an ascending ethanol series (70%, 90%, and 100% ethanol for a few minutes at each concentration), and dried in air. To generate a unique spectral signature for each chromosome, FISH probes were obtained by high-resolution flow sorting of individual mouse chromosomes, amplification of the chromosomal DNA by two rounds of depth of penetration-polymerase chain reaction (PCR), and labeling of the generated PCR products in a combinatorial manner with three fluorochromes and two haptens. The fluorochromes Spectrum Orange (deoxyuridine triphosphate [dUTP] conjugate; Vysis, Downers Grove, IL), rhodamine 110 (Perkin Elmer, Foster City, CA), and Texas red (12-dUTP conjugate; Molecular Probes, Eugene, OR) were used for direct labeling, and the haptens biotin-16-dUTP and digoxigenin-11-dUTP (Boehr-

FIGURE 1

Visualization of interconnecting chromosomes after treatment with OA during the G1 and G2 phases of the cell cycle. (**A** and **B**), Visualization of chromosomes during the G2 phase. (**C**), Visualization of chromosomes during the G1 phase. Stained with Giemsa. Original magnification, ×600.

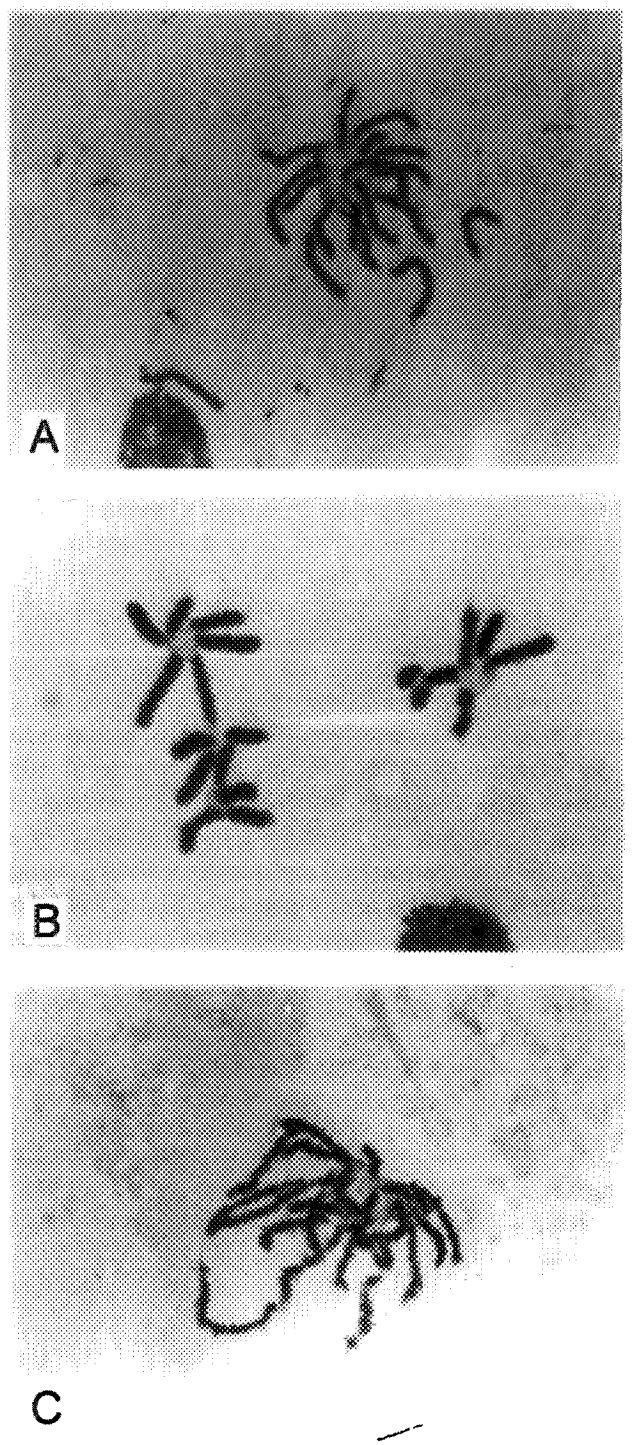

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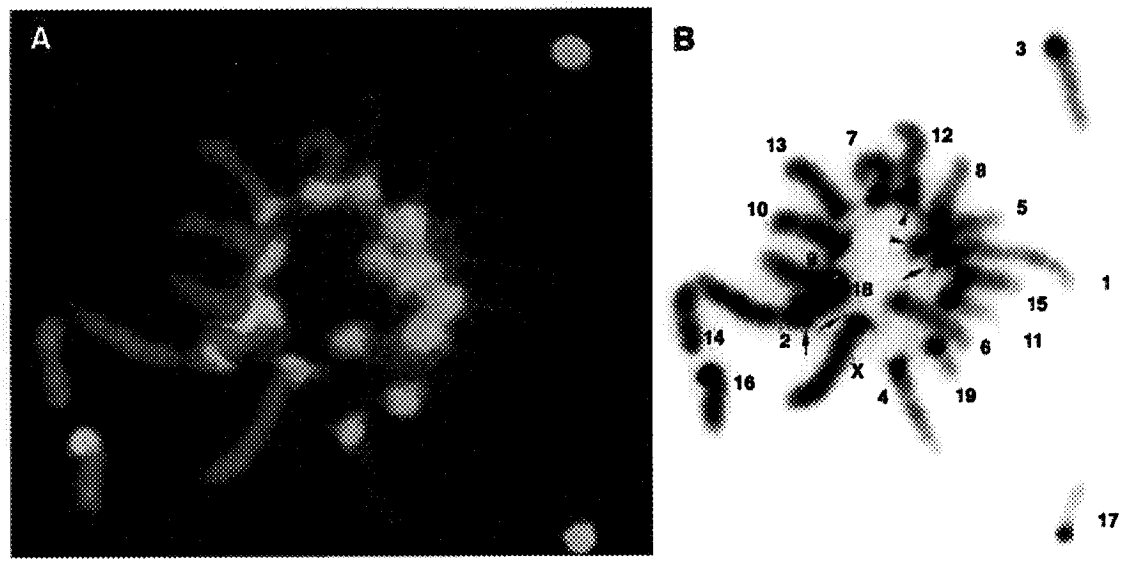
FIGURE 2

(**A**), In situ hybridization of chromosomes in the structure with major satellite DNA (green). (**B**), C-banding of interconnecting chromosomes in the structure. Stained with DAPI. Original magnification, ×600.



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inger Mannheim, Indianapolis, IN) were used for indirect labeling.

The probes were precipitated in the presence of an excess of mouse Cot-1 DNA (GIBCO BRL), resuspended in 50% formamide, added to an equal volume of 10% dextran sulfate in 2× SSC, denatured for 7 minutes at 88°C, and finally preannealed for 2 hours at 37°C. After hybridization, which was performed under sealed coverslips in a humidified chamber for 3 nights at 37°C, and a washing step, which was done three times each in 50% formamide/2× SSC, 1× SSC, and 4× SSC/Tween 20, biotin was detected by avidin-Cy5 (Amersham, Arlington Heights, IL) and digoxigenin-11-dUTP was detected by mouse anti-digoxigenin (Sigma, St. Louis, MO) followed by sheep antimouse Cy5.5 (Amersham).

The chromosomes were counterstained with diaminophenylindole (DAPI) and embedded in an antifade solution containing 1,4-phenylene-diamine (Sigma). Spectral images were acquired on a Leica DMRBE epifluorescence microscope equipped with an SD 200 SpectraCube (Applied Spectral Imaging) and a customized triple-bandpass optical filter (SKY1; Chroma Technology). Spectrum-based classification of the raw special images was performed using the software Sky View (Applied Spectral Imaging). Diaminophenylindole-banded gray-scale images of the same cells were captured separately, electronically inverted, and contrast-enhanced using the same software.

Mouse Major Satellite FISH Analysis

For a detailed explanation, see the article by Weier et al. (13). Probes were generated by PCR with the primers WGS1 and WGS2 5′-CCCAAGCTTGAAATGTCCACT-3′ and 5′-CCCAAGCTTTTTCTTGCCATA-3′, respectively. A second PCR incorporating 0.2 mM of biotin-11-dUTP was used to label the amplification product. Approximately 20 μg of probe DNA was precipitated with sodium acetate and ethanol, resuspended in 50% formamide, and added to an equal volume of 10% dextran sulfate in 2× SSC. This probe mixture then was denatured and added to slides that were processed as described for SKY.

Slides were allowed to hybridize for 3 nights, after which they were washed three times each in 50% formamide/2× SSC, 0.1× SSC, and 4× SSC/Tween 20. Detection of hapten-labeled sequences was performed by incubation with avidin-fluorescein isothiocyanate (Vector Laboratories, Inc.). Chromosomes were counterstained with DAPI and covered in 1,4-phenylenediamine antifade solution. Images were acquired on a Leica DMRBE epifluorescence microscope equipped with a cooled CCD camera (CH250; Photometrics).

Telomere FISH Analysis

Telomere-specific probes were prepared by PCR with the telomere repeat primer 5′=−(CCCTAA)$_7$−3′= in the presence of Spectrum Orange–labeled dUTP (Vysis, Downers Grove, IL). The resulting probe then was digested with deoxyribonuclease to yield a probe suitable for in situ hy-

FIGURE 3

In situ hybridization of chromosomes in the structure with telomeric DNA sequences. Original magnification, ×600.

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bridization (approximately 300–800 base pairs in length), precipitated, and resuspended in 50% formamide, 10% dextran sulfate, and 2× SSC. Approximately 50–100 ng of telomere repeat probe was used for each hybridization. After denaturation for 5 minutes at 80°C, hybridization with telomere probes was performed overnight at 37°C to chromosomes on glass slides. Samples were counterstained with DAPI and images were captured and processed as described previously for major satellite DNA with appropriate filters.

Fluorescent Microscopy

Zygotes were stained with DAPI and observed in ultraviolet light using a Zeiss microscope (Zeiss, Oberkocher, Germany).

RESULTS

Approximately 5% of mouse zygotes fixed after 50 minutes of exposure to OA displayed chromosomes with different degrees of condensation attached to the nucleolus (Fig. 1A) or secondary nucleoli (Fig. 1B), forming a peculiar structure. This structure was observed only when pronuclei were still visible under the inverted microscope. It is of interest that chromosomal attachment was no longer observed in the absence of a pronuclear membrane. Approximately 40 minutes after exposure to OA, only decondensed chromatin was observed. To determine whether the structure could be visualized throughout interphase, mouse zygotes were exposed to OA during G1. Approximately 20 hours after the administration of hCG and 50 minutes after exposure to OA, but before the disappearance of the pronuclei, the structure was observed in approximately 3% of all pronuclei examined (Fig. 1C).

In most cases, the structure was observed in either the X- or Y-containing pronucleus; in a few cases, it was observed in both. Although most of the chromosomes participated in the structure, a few were found to be separated. To determine whether they became detached as a result of the fixation procedure, a more gentle fixation (2% formaldehyde) followed by Hoechst staining with fluorescent microscopy was used. This allowed us to confirm that all chromosomes were attached to the nucleolus (or nucleoli) and that those that were separated became detached during spreading on the slide.

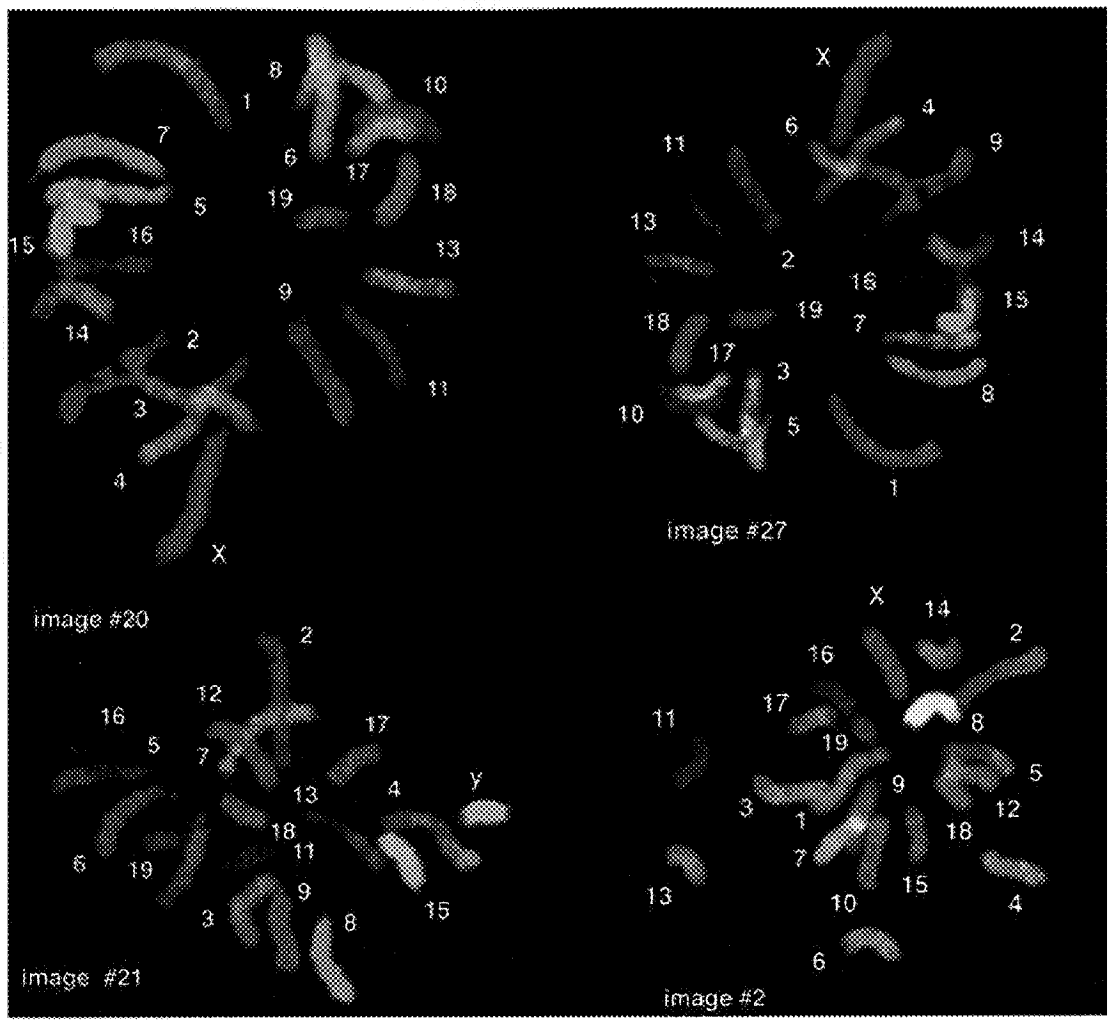
To verify that this chromosomal appearance was not an artifact induced by treatment with OA, the state of the chromatin at the end of the "natural" prophase was examined. The structure was indeed present during the natural cycle at approximately 28 hours after the administration of hCG. However, its existence apparently was very brief, so that without synchronization with OA, fewer than 1% of zygotes were found to have an identifiable structure at the end of prophase. There was still a large variation in the timing of the response, not only between individual zygotes but also between male and female pronuclei within one zygote, even in the presence of OA. This may explain the inability of other investigators to observe the phenomenon previously.

C-banding with DAPI revealed that in the structure, chromosomes were oriented to the nucleolus by their centromeric ends (Fig. 2B). Because the strands interconnecting the centromeric ends of chromosomes had an affinity for DAPI stain, we assumed that the chromosomes were connected by some type of DNA. Both telomeric and centromeric repeats were likely candidates for providing interchromosomal connection. Indeed, the most proximal part of the chromosomes to the structure were telomeres, which are known to form "sticky ends" and associations with each other (14). Because mouse chromosomes are acrocentric, however, centromeres also were located very close to the core of the structure, and thus direct centromeric association could not be excluded.

Figure 2A shows that mouse major satellite DNA overlapped with DAPI-stained DNA strands connecting centromeres of different chromosomes, indicating that chromosomal orientation was due to direct association of the centromere belonging to each chromosome. Unlike satellite DNA, telomeric sequences were completely condensed (Fig. 3) at this stage and did not interconnect chromosomes. Moreover, telomeric repeats were similar on both the proximal and distal ends of the chromosome; thus, telomere

FIGURE 4

Spectral karyotyping of chromosomes in the structures. Original magnification, ×600.



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attachment to this nucleolus would not provide a uniform inward chromosomal orientation by centromeres. These results indicated that all chromosomes within each individual pronucleus in a zygote were interconnected.

The observed interconnection of chromosomes presented a unique opportunity to determine the relative position of chromosomes during the interphase. Indeed, in the structure, chromosomes were condensed and could be identified reliably, whereas the order that existed during interphase was preserved by their interconnection.

Using the spectral karyotyping technique, up to 95% of the mouse chromosomes per nucleus could be identified reliably and simultaneously. Although some chromosomes, such as X and 1, tended to appear juxtaposed, the data (Fig. 4) could not systematically confirm colocalization for any chromosomal pair. This may be due to the three-dimensional nature of the structure, which tends to collapse onto itself.

DISCUSSION

Juxtaposition of the centromeres to nucleoli has been attributed to the localization of ribosomal gene clusters (also referred as nucleolus organizing regions, or NORs) in proximity to centromeres (3). However, NORs are not active in a zygote at the pronuclei stage (15). Moreover, the mouse genome has only six NORs, whereas our results show that all mouse chromosomes participate in the nucleolus through sharing of their satellite DNA. It is therefore reasonable to conclude that the nucleolus in a zygote is essentially a visualization of an interconnection between centromeres at the light microscopic level.

It is common knowledge that kinetochores that bind to centromeric sequences have affinity for microtubules, which are responsible for chromosomal movement at mitosis. During the interphase, kinetochores remain associated with centromeres in all somatic cells studied so far (16). Because, as our results show, centromeres remain condensed at the pronuclei stage, they are likely to retain an ability to attract microtubules. This might explain both syngamy and the often observed alignment of nucleoli from the opposing pronuclei. Therefore, we speculate that alignment of nucleoli may reflect intactness of the DNA, normal metabolism, and adequate microtubuli reserve in a good-quality zygote.

The apparent random association, and thus lack of chromosomal order, in the mouse nucleus is not surprising given that the chromosomes are connected directly by their almost identical satellite DNA (17). In contrast, the human satellite sequences are more diverse. For example, compared with other chromosomes, the satellite sequences of chromosomes 3, 4, 13, 14, 15, 21, and 22 are more similar (18). Thus, one would expect that these chromosomes would preferentially associate with one another. Although it would be less frequent, they would still associate with nonacrocentric chromosomes, as has been observed in human cells. Such a hierarchy of affinity between satellite sequences of different chromosomes resolves the illusive reality of chromosomal order in the interphase nucleus.

Our data show that satellite association ensures normal chromosomal division between cells. If, as data in the literature suggest, chromosomes remain associated throughout metaphase (19), this may ensure equal chromosomal division even if an individual chromosome becomes detached from the spindle. By itself, chromosomal order during the interphase most likely is not critical for other cell functions. This is consistent with the observation that, for instance, mice that carry a reciprocal robertsonian translocation are morphologically and functionally normal.

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